Development and Characterization of an Inducible Rat Model of Chronic Thromboembolic Pulmonary Hypertension

Paula-Anahi Arias-Loza,* Pius Jung,* Marco Abeßer, Sandra Umbenhauer, Tatjana Williams, Stefan Frantz, Kai Schuh, Theo Pelzer

Abstract—Chronic thromboembolic pulmonary hypertension (CTEPH) is an entity of PH that not only limits patients quality of life but also causes significant morbidity and mortality. The treatment of choice is pulmonary endarterectomy. However, numerous patients do not qualify for pulmonary endarterectomy or present with residual vasculopathy post pulmonary endarterectomy and require specific vasodilator treatment. Currently, there is no available specific small animal model of CTEPH that could serve as tool to identify targetable molecular pathways and to test new treatment options. Thus, we generated and standardized a rat model that not only resembles functional and histological features of CTEPH but also emulates thrombi fibrosis. The pulmonary embolism protocol consisted of 3 sequential tail vein injections of fibrinogen/collagen-covered polystyrene microspheres combined with thrombin and administered to 10-week-old male Wistar rats. After the third embolism, rats developed characteristic features of CTEPH including elevated right ventricular systolic pressure, right ventricular cardiomyocyte hypertrophy, pulmonary artery remodeling, increased serum brain natriuretic peptide levels, thrombi fibrosis, and formation of pulmonary cellular-fibrotic lesions. The current animal model seems suitable for detailed study of CTEPH pathophysiology and permits preclinical testing of new pharmacological therapies against CTEPH. (Hypertension. 2016;67:00-00. DOI: 10.1161/HYPERTENSIONAHA.116.07247.) • Online Data Supplement

Key Words: endarterectomy • hypertension, pulmonary • pulmonary embolism • rats, Wistar • thrombin

Chronic thromboembolic pulmonary hypertension (CTEPH) is an important but yet under-researched entity of PH.1 CTEPH is characterized by nonresolving thrombi that obstruct pulmonary arteries and promote progressive pulmonary vascular remodeling. The chronic increase in pulmonary vascular resistance translates into elevated pulmonary artery pressure and results in right ventricular (RV) hypertrophy, which ultimately causes RV dilatation and failure.2 The treatment of choice is pulmonary endarterectomy that consist in the surgical removal of organized thrombi to an extent that improves pulmonary hemodynamics. However, a relevant number of patients present persistent small-vessel vasculopathy post pulmonary endarterectomy,3 and many affected individuals are poor candidates for pulmonary endarterectomy because of a predominantly distally surgically inaccessible embolism pattern, high age, or severe comorbidities. Therefore to improve quality of life and eventually survival, numerous CTEPH patients require additional or alternative nonsurgical treatment. However, our understanding of the pathophysiology of CTEPH is limited and an obstacle in the development of new treatment strategies.4

The pathogenesis of CTEPH is still under debate. According to the local thrombosis hypothesis, CTEPH originates from primary distal pulmonary arteryopathy accompanied by secondary in situ thrombosis. However, this hypothesis does not provide a mechanism to explain proximal pulmonary artery thrombosis.5 Thus, the majority of experts in the field favor the embolism hypothesis that attributes CTEPH pathogenesis to a single or recurrent pulmonary embolisms (PE). The latter hypothesis is supported by a history of PE in ≥75% of all CTEPH patients and by the beneficial effects of timely performed pulmonary endarterectomy.2,6 Finally, several risk factors for CTEPH including specific procoagulation factors such as phospholipid antibodies, protein S and protein C, factor V mutations, malignancy, inflammatory bowel disease, and previous deep vein thrombosis are also risk factors for recurrent PE.6,7 It is not yet understood how PE progresses to CTEPH, and the mechanisms behind thrombi escape to thrombolysis and subsequent fibrotic transformation are still unknown. Consequently, there is a need for simple and reproducible CTEPH animal models that not only permit the analysis of molecular signals involved in the pathology of this disease but also enable the evaluation of new therapeutic strategies to improve the fate of individuals diagnosed with CTEPH.

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The generation of a small CTEPH animal model has proven to be a challenging task because of the efficient fibrinolysis, the considerable dilative reserve of the pulmonary vasculature, and the inability of the RV to overcome massive and acute increases in afterload. Previous animal models either involved complex surgical protocols in large animals or presented only some but not all features of human CTEPH. To address this issue, we established and standardized an easy to reproduce rat model that emulates pulmonary artery thrombosis and resembles functional and histological key features of CTEPH. This model overcomes endogenous thrombolysis by applying a thrombotic material that combines nondegradable polystyrene microspheres with a biologically active solution, which enables interactions of the pulmonary emboli with their local cellular environment. In addition, serial embolisms provide the RV with sufficient time to develop adaptive responses, such as cardiac myocyte hypertrophy.

Methods
The study was approved by the Lower Franconia Government. An expanded Materials and Methods section is provided in the online-only Data Supplement In short, 10-week-old male Wistar rats (Charles River, Germany) were randomized into sham (n=12) and PE (n=22) groups. Rats were weighed and anesthetized (1.5% isoflurane) before intervention. Pulmonary embolization was performed by 3 consecutive injections of a thrombogenic mixture (fibrinogen-/collagen-coated microspheres of 45-μm diameter—Distrilab 7545A—in saline solution with thrombin at 0.0027 U/μL) via the tail vein at a dosage of 1000 microspheres/g body weight (on weeks 10 and 11) and 750 microspheres/g body weight on week 12. Control animals underwent the same protocol but were treated with saline solution instead of the thrombotic material. Organ collection was performed after euthanasia under isoflurane anesthesia. RVs and lungs were prepared for histological analysis, and pieces of the right lung lobe were used for RNA reverse transcriptase polymerase chain reaction array analysis and Western blots.

Statistical Analysis
All data are expressed as means±SEM. Two-group comparisons were done by Student t test, P<0.05 were considered significant. Reverse transcriptase polymerase chain reaction array analysis was performed in the manufacturer’s integrated Web-based software package using ΔΔCt based fold-change calculations (SA Bioscences-Qiagen).

Results
Thrombotic Material
Numerous and different combinations of microspheres, collagen, fibrinogen, and thrombin were evaluated, ex vivo, to find a thrombotic material easy to inject, via catheter, into a rat tail vein. The final mixture used throughout the study consisted of 0.9% saline solution containing 5% microspheres, 5 mg/mL fibrinogen, 5.67 μg/mL collagen, and 0.0027 U/μL thrombin (Figure 1).

PE and Mortality
To prevent rats from going into cardiogenic shock on acute PE, we applied mechanical ventilation and carefully titrated the dose of microspheres to achieve submassive experimental PE without electrocardiographic signs of RV ischemia. After dose titration studies, we decided on administering 1000 microspheres/g body weight for the first and the second PE; the third PE included a dose reduction to 750 microspheres/g body weight. Sequential experimental PEs were performed at weekly intervals. The overall mortality was 27% and occurred during acute PE.

At the time of organ collection, the thrombotic material was detectable in the lungs of PE rats and histologically identified by the deposition of microspheres in the pulmonary arterial vasculature. Thrombotic material was cell and collagen enriched as evidenced by positive staining for nuclei (4′,6-diamino-2-phenylindole [DAPI]) and deposition of collagen (picrosirius red staining) (Figure 1).

Lung Histomorphology
The current protocol permitted us to generate a homogeneous extent of PEs among individual animals; the total number of microspheres per complete transversal lung section proved to be similar between rats (Table 1). The microspheres preferentially localized into distal pulmonary arteries and were distributed either as small (1–2
The distribution pattern into smaller and larger groups was similar among animals (Table 1).

PE also resulted in pulmonary vascular hypertrophy of small (<50-μm diameter) and mid caliber (between 50- and 150-μm diameter) pulmonary arteries (Table 1; Figure 2). The number of hypertrophied blood vessels of both calibers with and without lumen did not differ among PE animals (Table 1).

In addition, a perivascular cellular infiltration of a mixed population of CD45-positive cells including polymorphonuclear cells, macrophages, and mononuclear leucocytes was detected in lungs of PE rats (Figure 3; Figure S1 in the online-only Data Supplement). The lungs of these animals also presented cellular-fibrotic lesions (Figure 4), characterized as fibrotic-cellular agglomerations that could include blood vessels and microspheres.

Extensive histological analysis of brain, liver, kidney, as well as left and RV samples showed not only the absence of microspheres but also the lack of perivascular infiltrates in all tissues and organs (Figures S2 and S3). Likewise, no increase in serum markers of systemic inflammation was observed (C-reactive protein and interleukin 1β-1F2; Table 1).

### Table 1. Morphological, Hemodynamic, and Histological Data

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sham</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>404±8</td>
<td>390±6</td>
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<tr>
<td>Heart weight, mg</td>
<td>1110±28</td>
<td>1185±24*</td>
</tr>
<tr>
<td>LV+septum weight, mg</td>
<td>781±28</td>
<td>793±19</td>
</tr>
<tr>
<td>RV weight mg</td>
<td>213±5</td>
<td>269±9*</td>
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<tr>
<td>RV/(LV+septum) weight, mg/mg</td>
<td>0.28±0.01</td>
<td>0.34±0.01*</td>
</tr>
<tr>
<td>Echocardiographic analysis</td>
<td>n=4</td>
<td>n=7</td>
</tr>
<tr>
<td>TAPSE, mm</td>
<td>2.5±0.2</td>
<td>1.7±0.1*</td>
</tr>
<tr>
<td>Hemodynamics</td>
<td>n=12</td>
<td>n=15</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>134±4</td>
<td>125±1</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>366±11</td>
<td>384±8</td>
</tr>
<tr>
<td>RVSP, mmHg</td>
<td>25±0.8</td>
<td>32±1.2*</td>
</tr>
<tr>
<td>Serum ELISA</td>
<td>n=12</td>
<td>n=16</td>
</tr>
<tr>
<td>BNP, ng/mL</td>
<td>1.17±0.03</td>
<td>2.12±0.26*</td>
</tr>
<tr>
<td>Endothelin 1, pg/mL</td>
<td>2.0±0.3</td>
<td>3.1±0.5†</td>
</tr>
<tr>
<td>CRP, μg/mL</td>
<td>536±10</td>
<td>506±11</td>
</tr>
<tr>
<td>IL-1β/IL-1F2, pg/mL</td>
<td>0.10±0.01</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>Lung histomorphology</td>
<td>n=12</td>
<td>n=16</td>
</tr>
<tr>
<td>No. of microspheres</td>
<td>0±0</td>
<td>545±51*</td>
</tr>
<tr>
<td>No. of small groups of microspheres</td>
<td>0±0</td>
<td>208±14*</td>
</tr>
<tr>
<td>No. of big groups of microspheres</td>
<td>0±0</td>
<td>71±7†</td>
</tr>
<tr>
<td>No. of small caliber hypertrophied vessels with lumen</td>
<td>4.6±0.9</td>
<td>33.0±2.4*</td>
</tr>
<tr>
<td>No. of small caliber hypertrophied vessels without lumen</td>
<td>5.0±1.3</td>
<td>59.5±6.7*</td>
</tr>
<tr>
<td>No. of mid caliber hypertrophied vessels with lumen</td>
<td>2.2±0.9</td>
<td>21.4±3.5*</td>
</tr>
<tr>
<td>No. of mid caliber hypertrophied vessels without lumen</td>
<td>0.4±0.3</td>
<td>4.1±1.1*</td>
</tr>
<tr>
<td>No. of blood vessels with perivascular infiltration</td>
<td>14±7.8</td>
<td>68.4±17.1*</td>
</tr>
<tr>
<td>No. of cellular-fibrotic lesions with or without microspheres</td>
<td>0±0</td>
<td>10.4±2.5*</td>
</tr>
</tbody>
</table>

Values for lung histomorphology were quantified in a complete 10-μm transversal section of the left lung lobe. Values are presented as mean±SEM. BNP indicates brain natriuretic peptide; CRP, C-reactive protein; HR, heart rate; IL, interleukin; LV, left ventricle; PE, pulmonary embolization; RV, right ventricle; RVSP, right ventricular systolic pressure; and TAPSE, tricuspid annular plane systolic excursion.

*P<0.051 sham vs PE (2-tailed t test).
†P<0.051 sham vs PE (1-tailed t test).

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Reverse transcriptase polymerase chain reaction array analysis identified 4 differentially expressed mRNA transcripts at a fold regulation threshold ≥2.0 and P<0.05 after PE. Among these transcripts, Chemokine (C-C motif) ligand 12 (Ccl12) and matrix metallopeptidase 9 were more abundant in lung tissue of PE versus sham animals. In contrast, heat shock 70 kD protein 1B and heat shock protein 4-like mRNAs were significantly less abundant in lung tissue of PE when compared with sham animals (Table 2). At protein level, PE resulted in decreased expression and Ser1177-phosphorylation of eNOS and enhanced expression of gp91phox (Figure S4).

### Cardiac Physiology and Morphology

In comparison with sham rats, serial PE significantly increased RV systolic pressure and RV mass. RV hypertrophy, in PE compared with sham rats, was evident not only from increased RV weight (Table 1) but also from significantly enlarged RV cardiomyocyte cross-sectional areas (Figure S5). At the structural level, PE was associated with a modest but significant increase in RV collagen content (Figure S6). functionally, tricuspid annular plane systolic excursion was significantly lower in PE than in sham rats and was accompanied by higher brain natriuretic peptide and endothelin I serum levels (Table 1).
Discussion

We report a novel, technically simple and reliable rodent model of CTEPH. In this model, serial PEs resemble many key features of CTEPH including clot persistence and fibrosis, increased RV systolic pressure, RV remodeling, distal pulmonary artery hypertrophy, perivascular inflammation, and appearance of cellular-fibrotic lesions. In the past, different substrates had been used to induce acute or chronic PE including autologous clots, n-butyl-2-cyanoacrylate, and beads of other solid materials, administered to rodents, dogs, sheep, and piglets. Although most of these models present with clinical features of acute PE, only the work by Mercier et al. shows data on long-term pulmonary vascular hypertrophy, RV remodeling, and PH. Yet a valid animal model, the complex surgical approach, and the use of a large animal model limit its general and widespread use. Our study is thus the first to report a rodent model that not only resembles CTEPH but also is much easier to establish and maintain. The extent of PE can be quantified postmortem by simply counting the microspheres located in the middle of microspheres and the arrow points to a small blood vessel.

Figure 3. Pulmonary embolization resulted in perivascular cellular infiltration (B and D) of polymorphonuclear cells (1 in D), mononuclear leukocytes (2 in D), and macrophages (3 in D). A and C, Representative sections from sham animals. Hematoxylin & eosin staining. Scale bars, 50 μm.

Table 2. Stress and Toxicity Regulated Transcripts

<table>
<thead>
<tr>
<th>Unigene</th>
<th>Symbol</th>
<th>Description</th>
<th>Fold Regulation</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn.137780</td>
<td>Ccl12</td>
<td>Chemokine (C-C motif) ligand 12</td>
<td>3.249</td>
<td>0.011</td>
</tr>
<tr>
<td>Rn.1950</td>
<td>Hspa1b</td>
<td>Heat shock 70 kD protein 1B</td>
<td>−2.670</td>
<td>0.005</td>
</tr>
<tr>
<td>Rn.144829</td>
<td>Hspa4l</td>
<td>Heat shock protein 4-like</td>
<td>−2.194</td>
<td>0.015</td>
</tr>
<tr>
<td>Rn.10209</td>
<td>Mmp9</td>
<td>Matrix metallopeptidase 9</td>
<td>5.723</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Fold regulation represent the transcript regulation in the pulmonary embolism group in comparison with the sham group.

Figure 4. The pathology of pulmonary embolization included the presence of fibrotic/cellular lesions (B, C, E, and F). Cellularity is shown in hematoxylin & eosin staining (B and C) and fibrosis is shown as positive picrosirius red staining (E and F). The majority of these lesions contained microspheres (C and F). Normal lung histology of a sham animal is shown in A and D. Asterisks are located in the middle of microspheres and the arrow points to a small blood vessel.

also results in fibrosis and provides a biological scaffold to study the role of different clot components in vascular remodeling. Assuming that the current phenotype does not represent the maximum extent of RV and pulmonary vascular pathology, addition of other defined clot components (ie, fibrinogen mutant variants, coagulation factors, antibodies, and chemokines) offers the opportunity to study the resulting phenotype and the role of clot components in CTEPH development.

Previous studies state that an abrupt increase of RV afterload, because of massive PE, causes acute RV dilatation and failure. The result is thinning and scarring of the free RV wall, which is not a morphological feature of human CTEPH. We aimed to avoid this phenotype by inducing moderate but progressive chronic RV stress by repetitive PE with a dose of microspheres that does not result in sustained electrophysiographic alterations. In contrast to previous studies, we modified the biologically inert polystyrene microspheres by applying a biological covering that consisted of a fibrinogen and collagen mixture. The rationale was to emulate potential interactions of autologous thrombi with the cellular environment. Thus, we decided to use proteins known to be components of pulmonary emboli, including fibrinogen (major protein component of pulmonary emboli in humans and collagen. The latter is exposed after endothelial cells denudation and is known to promote platelet adhesion and activation. Thrombin was added to the bead slurry ex vivo to prime fibrinogen cleavage into fibrin and induce clotting. Together, the mixture emulates the last steps of the blood clotting cascade, when thrombin cleaves fibrinogen hereby inducing fibrin polymerization and clotting. Because thrombin remained active after injection into the animals, it is conceivable that thrombin and fibrin might have promoted activation and adhesion of additional blood components to the clot.

We chose the caudal vein administration route because of its easy accessibility for serial embolisms and the anatomy of the circulatory system of the rat. In the rat, venous blood flow is directed from the caudal veins via the inferior vena cava directly to the right atrium thus bypassing the portal circulation. Therefore, this administration route assures that the pulmonary arteries are the first ramified arterial tree filter retaining the thrombotic material and decreases the risk of nonpulmonary bead deposition. In support of this concept, microspheres were neither observed in brain, liver, kidneys, spleen, nor right and left ventricular tissue. Finally, the chosen
size of the microspheres fits the diameter of pulmonary pre-
capillary arteries, preventing leakage into the systemic
circulation.

The pulmonary pathology of our animal model included
distal pulmonary artery hypertrophy, fibrotic-thrombotic
material in pulmonary arteries, and perivascular cellular
infiltration. This phenotype is consistent with lung pathology
observed in CTEPH patients. In addition, we noted the
formation of cellular-fibrotic lesions, some of them contain-
ing microspheres and internal vascular channels. Because of
the morphology of these lesions, they may represent fibrotic-
endothelial thrombotic material with small-vessel revascularization, as
observed in fibrotic thrombi in human CTEPH. However, the
structure of these lesions is also analogous to human plexi-
form lesions, and further studies using labeled collagen and/or labeled fibrinogen are required to assess the origin of
these fibrotic-cellular lesions.

The lungs of PE rats also exhibited perivascular cellular
infiltration, a characteristic that is consistent with the presen-
tance of inflammatory cells in the pulmonary vascular wall
of CTEPH patients. Similar to what has been reported in
human CTEPH, cells infiltrating around pulmonary arteries
stained positive for CD45. The functional role of inflamma-
tory processes in the development of PH is not yet fully
understood but seems to be important because its inhibition confers
favorable effects against pulmonary vascular remodeling in
animal and human PH. Thus, the current animal model could
be used to identify PE-induced immune signals and evaluate
their role in pulmonary vascular remodeling. Therefore, it is
interesting to note that mRNAs encoding the potent chemot-
tractant for peripheral blood monocytes Ccl12 (monocyte che-
moattractant protein-5) were upregulated in PE lung material.
In line with this observation, some of the infiltrating mono-
nuclear cells stained positive for the inflammatory monocytes
marker Ly6C (Figure S1). Another transcript upregulated in
lung tissue of PE rats was matrix metalloproteinase 9, which
has already been associated with pulmonary remodeling and
formation of plexiform lesions including sprouting of vascular
channels. Finally, CTEPH pathology also involves a pro-
fibrotic cellular compartment; therefore, it is interesting that
the 2 downregulated transcripts (heat shock 70 kD protein 1B
and heat shock protein 4-like mRNA) have been associated
with lung cancer and development.

Limitations
The limited time frame of this study resulted in mild RV
hypertrophy without signs of RV failure. Although not the
focus of this study, additional rounds and careful titration of
bead dosage would be required to generate more extensive
RV hypertrophy and ultimately RV failure. Quantification
of pulmonary artery hypertrophy was based on the absolute
number of blood vessels fitting into a defined descriptive cri-
terion (increased number of media layers with decreased inner
luminal area). This quantification strategy does not normalize
against the sham group and therefore some pulmonary arteries
in the sham group were classified as hypertrophied.

Pulmonary artery stiffness is an important predictor of
mortality in patients with PH; thus a limitation of this study is
that we did not measure mechanical properties of pulmonary
arteries. However, we did observe pulmonary alterations in
the expression of proteins related to vascular function, including
a reduced expression and phosphorylation of the NO generat-
ing enzyme eNOS and an increased expression of the oxida-
tive stress marker gp91phox (Figure S4). Considering that NO
plays an important role in vasodilatation and gp91phox has
been shown to mediate pulmonary artery constrictor response
to endothelin I in hypoxia PH is possible that PE rats present
an altered pulmonary vasomotion. Therefore, further studies
on the role of chronic PE in oxidative stress, NO generation,
and pulmonary arteries contractility may provide important
insights into CTEPH pathology.

In conclusion, we generated a small animal model resem-
bling several key features of CTEPH that could not only be
used to study the pathophysiology of CTEPH but might serve
to evaluate novel treatment strategies such as novel tyrosine
kinase inhibitors for human CTEPH.

Perspectives
We have generated and evaluated a small rodent model of
CTEPH that permits quantification and characterization of
pulmonary damage and RV hypertrophy after chronic PE.
Our rat CTEPH model provides the opportunity to evaluate
the functional role of specific thrombi components on thrombi
fibrosis and distal vascular remodeling. Beyond this, it rep-resents a unique small animal model for preclinical studies
aimed to identify molecular signaling pathways relevant to
CTEPH progression. Finally, it facilitates cost-efficient therapeu-
tic studies on novel targets and candidate compounds for
nonsurgical treatment of CTEPH.

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Loza received travel expenses from Bayer Healthcare.

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None.

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Novelty and Significance

**What Is New?**

- We have developed a novel rodent model of chronic thromboembolic pulmonary hypertension that resembles key features of the disease including, decreased tricuspid annular plane systolic excursion, increased serum brain natriuretic peptide, pulmonary artery hypertrophy, thrombi fibrosis, perivascular pulmonary cellular infiltration, right ventricular hypertrophy and elevated right ventricular systolic pressure.

**What Is Relevant?**

- The first-time use of inert microspheres covered with fibrinogen/collagen/thrombin that serves to overcome thrombolysis and provides a bio-logically active scaffold that promotes local vascular fibrosis and adverse remodeling. The resulting phenotype provides novel opportunities to study not only the molecular mechanisms and possible treatment strategies of human chronic thromboembolic pulmonary hypertension but also the role of specific clot components that can be added to the emboli in a defined manner.

**Summary**

We described a new small rodent model of chronic thromboembolic pulmonary hypertension that resembles key features of the human disease including: clot persistence and fibrosis, increased right ventricular pressure, right ventricular remodeling, distal pulmonary arterial hypertrophy, and perivascular infiltration. This model will allow the test of new pharmacological approaches against chronic thromboembolic pulmonary hypertension and will permit to evaluate the role of the different components of thrombi in the pathology of the disease.
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DEVELOPMENT AND CHARACTERIZATION OF AN INDUCIBLE RAT MODEL OF
CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

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Short title: Rodent CTEPH model

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Extended methods

Rat model of CTEPH

This study was carried out in accordance with the recommendations “Guide for the Care and Use of Laboratory Animals” of the University Clinic Wuerzburg. The protocol was approved by the local government of Lower Frankonia ( Permit Number: 01-45/10). All in vivo experiments were performed under isofluoran pre-anesthesia (3%) and anesthesia (1.5 to 2%). All possible efforts were made to minimize suffering of the animals.

Preparation of pulmonary emboli material

One day before the embolism, human fibrinogen Sigma cat # F3879-1G was diluted in saline solution at a concentration of 10 mg/ml. The mixture was exposed to cold ultra-sonication, until no gel particles were left. The solution was sterile filtered through a 0.22 µm filter. Afterwards sterile collagen (Type I from rat tail Sigma Cat# C3867) to a 11.35 µg/ml final concentration was added to the fibrinogen solution (this solution was used within 48h of preparation). Equal volumes of polystyrene microspheres (45 µm diameter - Distrilab 7545A in 10% solution) and the fibrinogen/collagen solution were mixed and incubated overnight at 4°C (this mixture was used within 24h of preparation). The final microspheres concentration in the thrombogenic material was 0.998 million beads/ml.

In order to perform reproducible PE it is recommended to prepare single animal dose aliquots of the pulmonary emboli material, consisting in 500 µl of fibrinogen/collagen solution with 500 µl of polystyrene microspheres solution. To test correct preparation an aliquot of the thrombotic material should be mixed with thrombin from rat plasma (Sigma Cat# T5772 to a final concentration of 0.0027 U/µl); the thrombotic material should form a gel within 30 second upon addition of thrombin. It is recommended to perform ex-vivo tests to accustom to the speed necessary to achieve a proper load of the thrombotic material in the syringe and injection through the catheter (should be performed before clotting is completed), no concentrated pellet of microspheres should be left in the syringe or in the catheter after injection.

Pulmonary Embolization

Nine weeks old male Wistar rats (Charles Rivers, Germany) were randomized in 2 groups: pulmonary embolism (PE n= 22) and sham injection (sham n= 12). One week later the animals were weighted and set under isofluoran anesthesia by mechanical ventilation (100 % oxygen at ambient pressure). The microspheres-containing solution was warmed to room temperature and the required amount of microspheres needed for a dose of 1000 microspheres/g body weight was calculated. A 24G catheter was inserted in a tail vein (Prior to embolization the empty space in the catheter was measured ~100 µl- and was added to the volume of the thrombotic material needed for the embolism). Thrombin from rat plasma (Sigma Cat# T5772) was added to the fibrinogen/collagen coated microspheres to a final concentration of 0.0027 U/µl. The mixture was then injected via the tail vein catheter. Cardiac function was continuously monitored by ECG. Mechanical ventilation and anesthesia were continued until a stable ECG was observed. One week later, a second embolism was performed under identical conditions. 14 days after the first embolization the rats received a third embolism under identical conditions except that the amount of injected microspheres was reduced to 750 microspheres/g body
weight. Sham animals underwent exactly the same protocol but 0.9% saline was injected instead of microspheres containing thrombotic material. Seven days after the last pulmonary embolism / sham embolism, echocardiography was performed under isoflurana anesthet the (2%). Two days later, the rats underwent hemodynamic analysis (isoflurana 3% pre-anesthesia and 1.5% anesthesia). Immediately thereafter, rats were euthanized under isoflurana anesthesia and whole blood was collected and centrifuged to obtain serum. Serum was kept at -80°C until measurements of BNP (Bachem), ET-1 (Abcam), CRP (BD Biosciences) and IL-1 beta/IL-1F2 (R&D Systems) by EIA. Heart and lung were excised in a block, connective tissue was trimmed away and organ mass was measured. The left lung lobe was perfused with a Tissue-Tek/saline solution and fixation was continued overnight in Tissue-Tek solution at 4°C. The right ventricle, left ventricle and remaining lung tissues were kept at -80°C with Tissue-Tek as a preservative.

Cardiac histomorphology

Longitudinal 30 µm sections of the right ventricle were stained with wheat germ agglutinin (Alexa fluor 594 conjugate - Invitrogen Cat. Nr. w11262) or Picrosirius red (Sigma-Aldrich). Wheat agglutinin stained sections were used to measure cardiomyocyte cross sectional area by manual tracing the area of 100 individual cardiomyocytes in the ImageJ software. Collagen accumulation was estimated as the Picrosirius red positive staining area normalized against the total stained area (Keyence, BZ image analysis).

Lung histomorphology

After overnight incubation, in Tissue-Tek/saline solution, the left lung lobes were frozen in Tissue-Tek at -80°C. Transversal mid-sections were used to prepare 10 and 30 µm whole lung cryosections. The sections were stained with HE, Picrosirius red and DAPI according to standard protocols. Microphotographs of complete left lung transversal sections stained with HE were obtained and used to count the number of microspheres, hypertrophied blood vessels (with and without lumen), vessels with perivascular infiltration and cellular/fibrotic lesions. Additional lung sections were used for immunofluorescence staining of CD45 (Santa Cruz, sc-53047) and Ly6C (Santa Cruz, sc-134471). Donkey anti-mouse or anti-rabbit IgG Alexa Fluor 488 conjugates were employed as secondary antibodies (Invitrogen). DAPI (Sigma-Aldrich) was used to stain nuclei. Negative control sections were performed for all staining and consisted in the omission of the first antibody.

RT-PCR Arrays

Frozen lung tissues were briefly washed in cold RNA later solution and were used for total RNA isolation using the RNaseasy fibrous tissue Mini kit (Qiagen). 1 µg of RNA was reverse transcribed with the RT2 First strand kit (Sabiosciences-Qiagen). Real-time PCR was performed on each sample using the RT2Profiler, PARN-003ZA that allows the evaluation of 84 genes related to the rat stress and toxicity in respect to 6 housekeeping genes.

Complete lists of the genes analyzed are available online at:

http://www.sabiosciences.com/ArrayList.php
Data analysis was performed using the manufacturer’s integrated web-based software package for the PCR Array System using ∆∆Ct based fold-change calculations, available online at:

http://www.sabiosciences.com/pcrarraydataanalysis.php

Reference organs histomorphology

The presence of microspheres or perivascular infiltration was evaluated in sections of brain, liver, kidney left ventricle and spleen stained with HE according to standard procedures.

Western Blots

Parts of the left lung lobe were briefly washed in cold saline solution and used to prepare protein extracts. Western blotting was performed according to standard protocols (Biorad Turboblot System) using the following primary antibodies: anti-eNOS (BD transduction Laboratories, 610297), anti-Phospho Ser1177 eNOS (Upstate, 07-428) and anti-gp91phox (BD transductions laboratories, 611415). Immunoreactive proteins were visualized by HRP-coupled antibodies (Amersham) and ECL. The ImageQuant software (Biometra) was used for densitometric analysis based on peak area; β-actin was employed as internal standard (Santa Cruz, sc-81178).

Statistics

All data are expressed as mean ± SEM. Two-group comparisons were done by Student’s t-test, p values ≤0.05 were considered significant.

Supplementary figures
Fig S1. Pulmonary embolism resulted in perivascular cellular infiltration (HE staining sections in B and C). The infiltrates included CD45 positive cells (E) and Ly6C positive cells (H). Groups of mononuclear infiltrates were absent in lung sections from sham animals (HE stained sections in A). No groups of CD45 (D) or Ly6C (G) positive cells were detected in sham animals. Negative controls are shown in F and I. Asterisks are located in the middle of a pulmonary vessel.
Fig S2. Histomorphological analysis of brain (A and F); liver (B and G); kidney (C and H); left ventricle (D and I) and spleen (E and J) sections failed to detect microspheres in rats receiving pulmonary embolization (F, G, H, I and J). Panels A, B, C, D and E show control sections from sham animals. HE staining.
Fig S3. Histomorphological analysis of brain (A and F); liver (B and G); kidney (C and H) and left ventricular (D and I) sections failed to show perivascular infiltrates in rats receiving pulmonary embolization (F, G, H and I). Panels A, B, C and D show corresponding tissues of sham animals. Leukocytes were detected only in spleen tissue (sham: E and pulmonary embolism: J). HE staining.
Fig S4. Pulmonary embolism altered the expression of markers of vascular dysfunction, including reduced pulmonary expression of eNOS (A) and reduced content of its active phosphorylation form (B, P-eNOS 1177), besides of increased expression of gp91 (C).
Fig S5. Right ventricular cardiac myocyte hypertrophy was evaluated measuring cardiac myocyte cross sectional area in sections stained with Alexa Fluor 350 Wheat Agglutinin. Pulmonary embolization (B) resulted in increased right ventricular cardiac myocytes cross sectional area compared to sham animals (A). Panel C represents quantified data from at least 12 animals per group. * p<0.05 sham vs PE (two-tailed t-test)
Fig S6. Right ventricular collagen accumulation was measured in sections stained with Picrosirius red. Pulmonary embolism (B) resulted in increased right ventricular collagen accumulation in comparison to sham animals (A). C presents data of at least 10 animals per group. † p<0.05 sham vs PE (one-tailed t-test).